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Metabolism of small multiple doses of (^{14}C) nicotine in the cat

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Summary

1. The distribution and metabolism of (2′-^{14}C) nicotine given as a series of small intravenous injections (4 μg/kg every 60 s for 20 min) have been studied in the cat.

2. Blood concentrations of (^{14}C) nicotine reached a maximum of 100 ng/ml at 20 min whereas blood concentrations of cotinine were maximal shortly afterwards.

3. The maximum concentration of nicotine in the blood was greater than that obtained by giving the same total dose as a continuous infusion ((4 μg/kg)/min for 20 min).

4. Urinary excretion accounted for 90% of the total multiple dose in 72 hours. After 24 h, however, only 2·5% of the radioactivity as (^{14}C) nicotine and 0·05% as (^{14}C) cotinine was excreted.

5. Gastric juice after 2 h contained significant amounts of activity which was almost entirely composed of (^{14}C) nicotine and (^{14}C) cotinine.

6. There were significant regional differences in the concentration of (^{14}C) nicotine in the brain; amounts were greatest in cerebral hemispheres and smallest in the spinal cord. The hypothalamus and thalamus contained significantly higher concentrations of (^{14}C) nicotine than the whole mid brain. (^{14}C) Cotinine concentrations were highest in the cerebellum.

Introduction

Methods are now available for the assay of nicotine and its metabolites in tissues and body fluids of many species including man. Only recently, however, have these techniques become sensitive enough for a study of nicotine metabolism in relation to the tobacco smoking habit. Gas chromatographic analysis of urine of smokers revealed nicotine and cotinine (McNiven, Raisinghani, Patashnik & Dorfman, 1965; Beckett & Triggs, 1966) and more recently Isaac & Rand (1969) using a more refined GLC technique have measured nicotine concentrations in the blood of dogs after intravenous injection of nicotine.

A radiochemical assay (Turner, 1969) has been used to investigate the distribution and metabolism of (2′-^{14}C) nicotine in the cat after a single intravenous injection of 40 μg/kg of the drug. Single injections of nicotine, however, do not strictly parallel the human smoking situation. A dose regime consisting of small repeated intravenous injections, namely 2 or 4 μg/kg every 30 or 60 s for 20 min more closely parallels the nicotine intake of a cigarette smoker who inhales the smoke which he

takes into his mouth (Armitage, Hall & Morrison, 1968). In the study described here the distribution of (^{14}C) nicotine and (^{14}C) cotinine in the cat has, therefore, been studied using this multiple dose regime and, in particular, the regional distribution of these compounds in the brain has been investigated.

Methods

Materials

(−)-(^{14}C) Nicotine hydrogen tartrate labelled in the 2′ position was synthesized in these laboratories by Dr. H. Roderick using the method of Decker & Sammeck (1964). Specific radioactivity of the product was 16–20 mCi/mmol. Radiochemical purity was constantly checked by thin layer and paper chromatography and by radioautography.

A sample of Demethylcotinine was generously given by Professor H. McKennis, jun., Richmond, Va., USA. Nicotine 1′-oxide was synthesized by Dr. H. Roderick and a sample of (−)-cotinine was obtained from Dr. Chesterfield of the Imperial Tobacco Group, Bristol. Nornicotine was obtained from Fluka AG, Chemische Fabrik, Buchs, Switzerland. All other reagents and solvents were of A. R. Grade.

Measurement of radioactivity

A Packard TriCarb Model 3375, liquid scintillation spectrometer (Packard Instruments Ltd., Wembley, Middlesex) was used for measurement of radioactivity. The liquid scintillator was that described by Evans (1961), namely dioxan-ethanol-xylene containing naphthalene, 2,5-diphenyl-oxazole and 1,4-*bis*-(4-methyl-5-phenyloxalzol-2-yl) benzene. In this system ^{14}C was measured with a maximum efficiency of 83%. Efficiency of radioactive measurements was determined by automatic external standard or the channels ratio method, samples being counted to at least 1% accuracy.

Chromatography

Labelled compounds in extracts of tissues or body fluids were separated by descending paper chromatography and thin layer chromatography on silica using the solvent systems described previously (Turner, 1969).

Radioautography

Paper and thin layer chromatograms were overlaid on Kodirex X-ray film (Kodak Ltd., London, WC2). Chromatograms and films were kept pressed together at room temperature for up to 4 weeks.

Animals

Experiments were performed on female cats weighing 2–2·5 kg and bred in these laboratories. Three cats were used for each determination of tissue and body fluid radioactivity. For studies on 24 h excretion of radioactivity, unanaesthetized cats were fitted with a small valve (Hall, Gomersall & Heneage, 1968) attached to the skull and connected to a cannula inserted in the left jugular vein. In experiments on cats anaesthetized with chloralose the procedures as described earlier were used (Turner, 1969). For studies on the distribution of (^{14}C) nicotine and (^{14}C) cotinine in brain, the tissue was dissected into regions as follows: the

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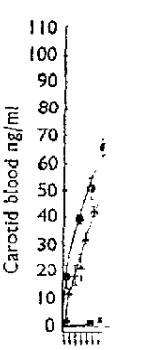

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cerebral hemispheres and cerebellum were removed; the remaining brain was further subdivided by section at the prepontine and spinal medullary levels; the tissues rostral to the prepontine section were assayed as a whole and are referred to as mid brain. In certain experiments thalamic and hypothalamic tissue was dissected out and each homogenized separately.

Total radioactivity of body fluids such as urine, bile and tissue extracts was measured directly without prior extraction. Samples of urine (100 μl) or bile (50 μl) or trichloracetic acid extracts of tissues (200 μl) were added to the vial which contained 15 ml scintillator. (^{14}C) Nicotine and (^{14}C) cotinine were extracted from tissue and body fluids by the methods previously described (Turner, 1969).

Identification of metabolites of (^{14}C) nicotine

Livers from anaesthetized animals killed 20 min after the start of intravenous injection of (^{14}C) nicotine were homogenized and extracted as described by Papadopoulos (1964). Pooled bile samples (4·2 ml), obtained by dissection of the gall bladders at the end of the experiments were concentrated to small volume (5 ml) *in vacuo* and a 1 ml portion quantitatively assayed for nicotine and cotinine. Pooled urines (3·5 litres) were concentrated similarly to 250 ml, filtered, and a portion eluted overnight from a small strip of Whatman No. 1 paper using an alkaline solvent system (Turner, 1969). The eluate was acidified with HCl and evaporated to dryness *in vacuo* at 40–50° C. The residue after evaporation of the eluate was dissolved in 15 ml water. Eighty per cent of the activity was recovered in the urine.

Results

Nicotine and cotinine in blood

The amounts of (^{14}C) nicotine and (^{14}C) cotinine appearing in carotid blood over a 2 h period after the start of the intravenous injections of (^{14}C) nicotine are shown in Fig. 1. The concentration of (^{14}C) nicotine rose steadily during the 20 min injection period and then immediately declined. The concentration declined to half the

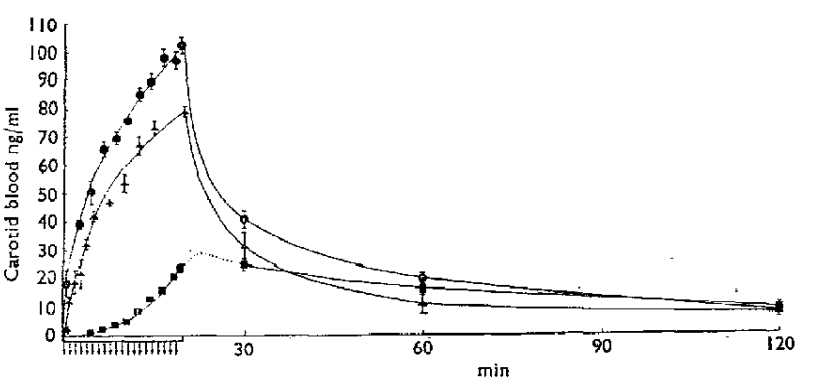

FIG. 1. Carotid blood concentrations of (^{14}C) nicotine and (^{14}C) cotinine over a 2 h period during and after intravenous injection of 4 μg of (^{14}C) nicotine/kg every minute for 20 min and after continuous intravenous infusion of (4 μg of (^{14}C) nicotine/kg)/min for 20 minutes. ●, Nicotine; ■, cotinine after multiple injection; ▲, nicotine after continuous infusion. Vertical bars represent the S.E.M.

peak value in 7·5 minutes. Cotinine concentrations which appeared in the blood after the third injection (2·5 min) rose at an increasing rate during the 20 min period, reaching a concentration of about 23 ng/ml after 20 minutes. The concentrations continued to rise during the next 10 min but the time at which maximum concentration occurred was not measured. After 30 min the concentrations of both (^{14}C) nicotine and (^{14}C) cotinine declined at similar rates, and after 2 h comprised approximately equal proportions (44·7 ± 0·8% nicotine and 48·2 ± 0·2% cotinine) of the extractable blood radioactivity.

Also shown in Fig. 1 are the nicotine concentrations obtained during and after a continuous infusion of (^{14}C) nicotine at a rate of (4 μg/kg)/min for 20 minutes. The concentration of nicotine again rose during the 20 min period but to a smaller maximum than had occurred with the multiple dose regime, the differences in the two maxima being statistically significant. (^{14}C) Cotinine concentrations though not shown in the figure, were determined and the shape of the time course curve was similar to that obtained during the multiple dose experiments. The concentration of cotinine 20 min after infusion was 19·0 ± 1·3 ng/ml and again the maximum concentration occurred at between 20 and 30 minutes.

Excretion of radioactivity in urine and faeces

Radioactivity in urine collected for 24 h periods over 72 h, from three unanaesthetized cats given 20 injections of 4 μg/kg (^{14}C) nicotine in 20 min, was measured by counting aliquots directly. Figure 2 shows the results. In 3 days 90% of the injected dose had been excreted, 77% being excreted in the first 24 hours. Results

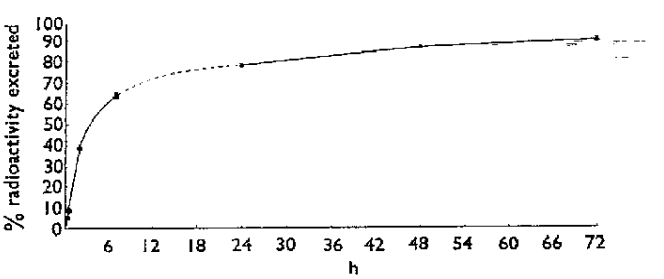

FIG. 2. Urinary excretion of radioactivity over a 72 h period after intravenous injection of 4 μg of (^{14}C) nicotine/kg every minute for 20 minutes. Vertical bars represent the S.E.M. ▲, Unanaesthetized animals; ●, anaesthetized animals.

TABLE 1. *The proportions of (^{14}C) nicotine and (^{14}C) cotinine excreted in urine and gastric juice relative to the sample activity and total dose of radioactivity expressed as the mean* ±S.E.M.

Time after start of injection	% Nicotine in urine	% Cotinine in urine	% Nicotine relative to total dose	% Cotinine relative to total dose
5 min	82·4 ± 0·6	0	0·19 ± 0·03	0
10 min	68·4 ± 1·4	0·63 ± 0·08	0·84 ± 0·05	0·007 ± 0·002
20 min	41·2 ± 1·0	0·63 ± 0·13	1·92 ± 0·31	0·025 ± 0·002
30 min	26·2 ± 2·1	0·47 ± 0·09	2·12 ± 0·25	0·037 ± 0·004
120 min	11·2 ± 1·8	0·78 ± 0·11	4·22 ± 0·46	0·300 ± 0·048
24 h	3·2 ± 0·1	0·07 ± 0·01	2·52 ± 0·09	0·054 ± 0·009
Gastric juice (2 h)	65·7 ± 5·2	27·9 ± 3·9	1·39 ± 0·68	0·53 ± 0·18

At each time interval three animals were killed and bladder contents collected.

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TABLE 2. (^{14}C) *Nicotine*

Spinal medulla	N
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Spinal cord	N
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Cerebellum	N
	C
Hind brain	N
	C
Mid brain	N
	C
Thalamus	N
	C
Hypothalamus	N
	C
Cerebral hemispheres	N
	C
Lung	N
	C
Liver	N
	C

* Significantly differe
† significantly different

obtained for the urinary excretion of radioactivity for periods of 20 min 7 h after the start of the injections in anaesthetized animals are shown in the same figure. Urine was obtained from these animals by removing the bladder contents when each animal was killed. In all experiments urinary pH was monitored and lay within the range 6·0–6·9. Twenty-four hour urine volumes were within the range 200–250 ml.

Qualitative studies on the excretion of (^{14}C) nicotine and its metabolites revealed all the compounds previously identified after a single injection of (^{14}C) nicotine, namely cotinine, demethyl cotinine, pyridyl acetic acid, nornicotine and nicotine-1′-oxide, in addition to four unidentified compounds. Results of quantitative studies on the urinary excretion of unchanged (^{14}C) nicotine and its metabolite (^{14}C) cotinine are shown in Table 1.

The greater proportions of the nicotine and cotinine appear to be excreted in 2 h, about 4·2% nicotine and 0·3% cotinine being found in urine at this time. Twenty-four hour collections of urine contained smaller proportions of the two compounds. (^{14}C) Nicotine appeared very rapidly in the urine, small but significant amounts being present in the bladder contents of animals 5 min after the start of injections. (^{14}C) Cotinine could not be detected at this time, however, though it was present in the 10 min urine samples. A large proportion of the urinary radioactivity obtained over 24 h—approximately 80%—was not extractable into methylene dichloride. Faeces collected over the first 24 h contained 0·71 ± 0·11% of the total injected dose of radioactivity.

Excretion of radioactivity into bile and gastric juice

Bile samples were obtained at the end of the experiment by dissection of the gall bladder and removal of the contents. All the samples contained radioactivity. Quantitative studies on radioactivity in pooled bile samples revealed (^{14}C) nicotine

TABLE 2. (^{14}C) *Nicotine (N) and* (^{14}C) *cotinine (C) in brain and other tissues expressed as ng/g wet weight of tissue* ± S.E.M.

		Time after start of injections				
		5 min	10 min	20 min	30 min	120 min
Spinal medulla	N	56·8 ± 1·9	100·9 ± 1·6	151·9 ± 2·1	52·8 ± 2·6	9·7 ± 0·1
	C	1·0 ± 1·0	3·8 ± 1·9	8·6 ± 1·0	7·8 ± 0·8	5·4 ± 0·3
Spinal cord	N			99·0 ± 4·1		11·9 ± 1·1
	C			3·3 ± 0·5		3·1 ± 0·8
Cerebellum	N	70·1 ± 10·8	121·5 ± 5·2	205·8 ± 8·0	64·2 ± 0·1	14·6 ± 1·2
	C	1·2 ± 0·6	2·6 ± 1·8	10·6 ± 1·0	9·4 ± 0·9	8·3 ± 0·6
Hind brain	N	59·5 ± 1·9	117·2 ± 2·8	172·3 ± 6·0	59·6 ± 3·2	14·1 ± 0·6
	C	0·6 ± 0·6	2·6 ± 1·6	5·3 ± 0·5	5·8 ± 1·0	4·3 ± 1·1
Mid brain	N	52·5 ± 1·9	93·5 ± 3·7	135·7 ± 5·6	53·5 ± 2·0	13·9 ± 0·5
	C	1·2 ± 0·6	2·4 ± 1·2	5·2 ± 0·9	5·3 ± 0·4	3·9 ± 0·2
Thalamus	N			202·3 ± 7·3*		
	C			5·2 ± 0·5		
Hypothalamus	N			155·6 ± 2·6‡		
	C			4·4 ± 0·4		
Cerebral hemispheres	N	88·5 ± 1·9	150·3 ± 4·5	251·1 ± 2·7†	89·9 ± 1·9	19·8 ± 0·2
	C	0·8 ± 0·8	2·0 ± 1·0	4·6 ± 0·4	7·8 ± 1·6	5·9 ± 0·8
Lung	N	67·7 ± 3·9	88·3 ± 2·3	123·1 ± 2·5	69·3 ± 7·8	18·7 ± 1·6
	C	6·1 ± 0·2	9·4 ± 0·9	15·1 ± 0·8	14·2 ± 0·9	6·3 ± 0·8
Liver	N	77·2 ± 0·2	99·2 ± 6·8	130·7 ± 4·0	101·6 ± 3·8	22·1 ± 0·7
	C	10·7 ± 2·7	34·0 ± 2·2	44·9 ± 4·5	35·7 ± 2·3	13·2 ± 0·5

* Significantly different from mid brain, $P < 0·01$; ‡ significantly different from mid brain, $P < 0·05$; † significantly different from cerebellum, $P < 0·01$.

as the greater component (51·7%). (^{14}C) cotinine comprising 4·1% of the activity. Gastric juice collected 2 h after the start of injections contained a significant amount of radioactivity (2·07±0·93%). Quantitative studies were performed on 1 ml portions of the juice and revealed (^{14}C) nicotine and (^{14}C) cotinine as the greater components, the proportions of the two compounds being shown in Table 1.

(^{14}C) Nicotine and its metabolites in brain and other tissues

The concentrations of (^{14}C) nicotine and (^{14}C) cotinine in various regions of the brain were measured over 2 h from the start of injections and the results are shown in Table 2. Also included in the table are the corresponding values for lung and liver. The amounts of nicotine and cotinine present are expressed as ng/g wet weight of tissue. Maximum amounts of (^{14}C) nicotine occurred in all tissues at the end of the 20 min injection period. (^{14}C) Cotinine concentrations in some brain regions, however, continued to rise until 30 minutes. The largest amount of nicotine occurred in the cerebral hemispheres and the smallest in the region of the spinal cord between C1 and C2. The mid brain section contained significantly smaller amounts of nicotine than the surrounding regions but thalamus and hypothalamus, dissected from the mid brain section, contained concentrations of nicotine which were significantly greater ($P<0·01$) than the remainder. Regional variation of cotinine concentrations in the brain occurred though the variations were slight. Relatively larger amounts of this metabolite occurred in the cerebellum at 20 min, however, and persisted for at least 2 hours.

The proportions of radioactivity representing (^{14}C) nicotine over the 2 h period are shown in Table 3. The brain regions as a whole still contained about 90% of the compound at the end of the 20 min injection period. Lung tissue contained a smaller amount of activity representing (^{14}C) nicotine than did liver. At all times both tissues contained the drug as a minor proportion of the radioactivity.

Chromatography coupled with radioautography of 20 min liver extracts revealed the same identifiable metabolites as described previously. No (^{14}C) nicotinic acid could be found. In addition, five unidentified radioactive spots were observed.

Discussion

The results described here provide quantitative evidence of the time course of the distribution and fate of nicotine in the cat, which may be of greater relevance to the human smoking situation than results of studies in which only a single injection of nicotine is given. Isaac & Rand (1969) have reported studies on the blood

TABLE 3. % *Radioactivity as* (^{14}C) *nicotine in regions of the brain and other tissues*±S.E.M.

	Time after start of injections				
	5 min	10 min	20 min	30 min	120 min
Spinal medulla	88·1±1·2	84·0±0·6	80·5±0·9	69·9±0·9	26·1±1·4
Spinal cord			89·6±1·3		29·5±0·3
Cerebellum	98·4±2·0	98·8±1·6	91·2±0·9	74·5±1·2	31·5±1·2
Hind brain	97·1±1·3	97·3±0·5	89·8±1·1	74·0±1·1	30·6±1·6
Mid brain	97·0±0·4	95·0±2·1	90·1±1·7	76·9±1·3	34·5±0·6
Thalamus			90·2±1·0		
Hypothalamus			89·0±1·0		
Cerebral hemispheres	98·9±1·1	98·8±2·2	92·2±1·0	73·5±1·9	35·4±0·9
Lung	33·5±0·4	25·1±0·6	21·8±1·2	20·5±0·6	11·9±1·1
Liver	45·5±1·6	32·7±3·5	29·9±1·7	22·6±1·1	15·0±1·0

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n	120 min
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	29·5±0·3
2	31·5±1·2
1	30·6±1·6
3	34·5±0·6
9	35·4±0·9
6	11·9±1·1
1	15·0±1·0

concentrations of nicotine in dogs after tobacco smoke was administered into the lungs. They observed a rise in blood nicotine following each puff of smoke, concentrations fluctuating markedly as might be expected. In our experiments blood was sampled 30 s after an injection and a gradual increase in the blood concentration of nicotine was observed over the 20 min period. A smooth curve has been drawn through the points to illustrate the general trend but it is realized that the overall picture will be more closely represented by a fluctuating graph. Cotinine concentrations, which were significant after 2·5 min, rose at an increasing rate reaching maximum between 20 and 30 minutes. (^{14}C) Nicotine concentrations declined after the multiple injections at a much slower rate than did blood concentrations after a single injection of nicotine ($T\frac{1}{2}$ of 7·5 min as compared with $T\frac{1}{2}$ of 1·5 min for the single dose). The fact that the multiple dose regime results in a larger maximum concentration of (^{14}C) nicotine in the blood than occurs when the same dose is given as a continuous infusion over the same period, is consistent with pharmacological observations (Armitage, Hall & Sellers, 1969).

Most of the injected radioactivity was excreted via the urine but only a small proportion was unchanged (^{14}C) nicotine and its metabolite (^{14}C) cotinine. Although the amount of nicotine excreted was higher than that excreted after a single intravenous dose in 24 h (Turner, 1969), the amount of cotinine was much smaller. Since 24 h urine volumes were of the same order (200–250 ml), and the pH range for these samples was similar in both series of experiments, the reason may lie in the fact that in our experiments all the animals were female whereas in the other experiments they were male. Beckett, Gorrod & Jenner (1969) have observed a similar sex difference in the urinary excretion of nicotine in human non-smokers. The observation that the amounts of the two compounds present in the 24 h urine were lower than those in the 2 h samples might suggest an effect of the chloralose anaesthetic though the indication that the graphs of total radioactivity excreted in anaesthetized and unanaesthetized cats (Fig. 2) appear to lie on the same curve does not support this. Because the bulk of the nicotine and cotinine appears in the urine during the first 2 h, it is possible, alternatively, that a significant percentage of the compounds could be reabsorbed during the remaining 22 h before 24 h collection.

The proportion of radioactivity remaining unaccounted for and possibly remaining in the animals after 3 days was about half that remaining after the same time in animals which received the single injection (Turner, 1969). Actual amounts of radioactivity remaining are similar in both cases, since the total dose given in our experiments is twice that of the single dose studies. The nature of this radioactivity is as yet unknown.

Gastric juice contained significant amounts of radioactivity after 2 h, the greater part of which was (^{14}C) nicotine and (^{14}C) cotinine. This finding is in accord with the studies of Andersson, Hansson & Schmiterlöw (1965) who observed a similar relative increase in the radioactivity of stomach content in mice, rats and cats after a single intravenous injection of larger amounts of (^{14}C) nicotine. The pH gradient between plasma and gastric juice could account for such an excretion, but it is also possible that swallowed saliva could contribute to the radioactivity in the stomach, since the salivary glands accumulate significant amounts of radioactivity (Appelgren, Hansson & Schmiterlöw, 1962), and nicotine can stimulate salivary flow (Larson, Haag & Silvette, 1961).

There were marked differences in the nicotine content of the various regions of the brain which were studied. The cerebral hemispheres contained significantly more (^{14}C) nicotine than the other regions, the region containing the smallest amount being that portion of the spinal cord between C1 and C2 where nicotine exerts a definite pharmacological effect in causing twitching of the ears (Armitage *et al.*, 1967). The mid brain region contained smaller concentrations of nicotine than did the surrounding brain tissue but significantly higher concentrations of the drug were observed in the hypothalamus and thalamus. These observations are consistent with the autoradiographic evidence from kitten brain of Schmiterlöw *et al.* (1967) who in addition observed a concentration of radioactivity (presumed to be largely nicotine) in the hippocampus.

In view of our interest in the effects of nicotine on the central nervous system and our finding that nicotine releases ^{3}H-noradrenaline from the hypothalamus (Hall & Turner, unpublished observations) the observation of drug concentration in this region relative to the surrounding tissue is noteworthy.

(^{14}C) Cotinine concentrations in the brain remained low throughout the period of study, the changes generally paralleling those occurring in blood. After 20 min, however, the cerebellum contained relatively larger amounts of cotinine than did other regions. Cotinine appears to penetrate into brain tissue slowly because the concentrations of cotinine were only slightly higher than might have been expected on the basis of blood concentrations alone.

In both liver and lung tissue the proportions of nicotine were much smaller than in brain. As expected, the concentrations of cotinine present in the liver were much higher than elsewhere, being maximal at the end of nicotine injections.

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